Biochimica et Biophysica Acta, 957 (1988) 237–242
Elsevier

BBA 33253

The reaction mechanism of bovine kidney biliverdin reductase

Elizabeth Rigney and Timothy J. Mantle

Department of Biochemistry, Trinity College, Dublin (Ireland)

(Received 27 May 1988)

Key words: Biliverdin reductase; Reaction mechanism; (Bovine kidney)

The steady-state kinetics of biliverdin reductase can be studied in detail at pH 9 as under these conditions the K_m for biliverdin is high enough to obtain reliable measurements of the initial rate in the absence of any biliverdin binding proteins. The initial rate kinetics and the product-inhibition studies are consistent with an ordered sequential mechanism provided the biliverdin concentration was below 20 μM. Above this concentration significant flux occurs through a substrate inhibition pathway involving an enzyme-NAD(P)-biliverdin complex. Chloride is shown to cause a significant activation of the enzyme under certain conditions and this is shown to result from an inability of biliverdin to bind to an enzyme-NAD-chloride complex.

Introduction

Biliverdin reductase is an NAD(P)-linked monomeric oxidoreductase which catalyses the reduction of biliverdin to produce the bile pigment, bilirubin. The enzyme is believed to have a function clearing biliverdin from the foetus [1] and recently the product bilirubin has been suggested to play a role as an antioxidant [2,3]. In most species the enzyme exists as a monomer of M_r 34 000 [4–6]; however, the guinea pig and hamster enzymes have M_r values that are approximately twice this value [7,8]. Biliverdin reductase is subject to potent substrate inhibition by biliverdin [9,6] although this effect is probably modulated in vivo by intracellular binding proteins [10]. There has been no detailed study of the reaction mechanism of biliverdin reductase, although it has been suggested that the enzyme obeys an ordered mechanism [7,6]. These studies were conducted in the presence of serum albumin which complicates the analysis by binding the substrate biliverdin [6] and also appears to have direct effects on the enzyme activity [11]. While it is highly desirable to analyse the reaction mechanism for biliverdin reductase in the absence of serum albumin this is not possible at a physiological pH, due to the insolubility of the product bilirubin and the low K_m for biliverdin. This last aspect is exacerbated using NADPH as the cofactor [7,11]. We have recently shown that biliverdin binding, both productively and non-productively, is less tight at alkaline pH values (Rigney and Mantle, unpublished data) and have utilised this observation to study the steady-state mechanism at pH 9. Details of this work are presented in the present report.

Materials and Methods

Materials

Biliverdin was synthesised by the method of McDonagh [12]. Biliverdin reductase was purified as described previously [8].

Methods

Enzyme assays. Biliverdin reductase activity was measured by following the production of bilirubin spectrophotometrically by monitoring the increase

Correspondence: T.J. Mantle, Department of Biochemistry, Trinity College, Dublin 2, Ireland.

in absorbance at 460 nm. The buffers used were: 0.1 M Tris-HCl/0.1 M sodium phosphate, and 0.02 M sodium pyrophosphate/0.2 M sodium phosphate at the pH values indicated. In the steady-state study the buffer used was 0.02 M sodium pyrphosphate/0.2 M sodium phosphate (pH 9). The absorption coefficient for bilirubin in this buffer at 460 nm is 54.7 $nM^{-1} \cdot cm^{-1}$ and for biliverdin the value is 4.08 $mM^{-1} \cdot cm^{-1}$ (Rigney, unpublished data). All assays were conducted in triplicate at 30 °C.

Treatment of data. All initial rate data were fitted to a rectangular hyperbola by the method of Wilkinson [13]. Slope and intercept replots were fitted by linear regression.

Results

Effect of pH

Biliverdin reductase was stable for at least 25 min in all of the buffer systems described in this work. The effect of pH on the NADPH- and NADH-dependent activities of biliverdin reductase are shown in Fig. 1. A pH optimum of 8.5 was found with NADPH when all three buffer systems described in the legend to Fig. 1 were used. The optimum pH observed when NADH was the cofactor was found to depend on the buffer system used. In sodium phosphate buffer the pH optimum is at 7 whereas in Tris-HCl the optimum shifts to a lower value. A similar phenomenon was observed with sodium pyrophosphate/sodium phosphate buffer in which case the pH optimum was 6. The effect of pH on the initial-rate kinetics with biliverdin as the variable substrate is shown in Fig. 2. This shows that the substrate inhibition is far more potent at acid and neutral pH values than at alkaline pH values. In addition the apparent K_m values for biliverdin are considerably higher at alkaline values than at acid or neutral values. At pH 9 it is therefore feasible to examine the steady-state kinetics of biliverdin reductase in the absence of serum albumin.

The initial rate kinetics of biliverdin reductase

A linear relationship was demonstrated between enzyme concentration and initial rate over the range 0.3–3.9 μg per assay. All kinetic experiments were conducted within this range. Initial rate measurements when the biliverdin concentration was held constant and the NADPH concentration varied (5–150 μM) yielded linear double-reciprocal plots (Fig. 3). When biliverdin was the variable substrate (0.5–10 μM) at fixed levels of NADPH, linear double-reciprocal plots were obtained (Fig. 3). The slopes and intercepts from each plot were replotted against the reciprocal of the fixed substrate concentration and the kinetic constants calculated by the method of Florini and Vestling [14]. These are shown in Table I for the cases when the data set were analysed with biliverdin as the variable substrate and NADPH was held constant at various fixed levels, and when NADPH was varied and the concentration of biliverdin was held constant at different fixed levels.

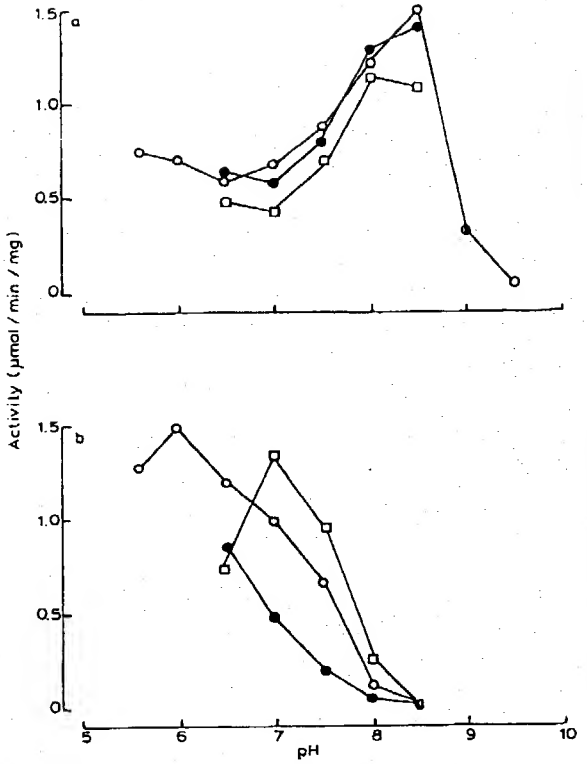

Fig. 1. The pH dependence of biliverdin reductase. Biliverdin reductase (1.95 μg) was assayed at 30 °C in 0.1 M Tris-HCl (●); 0.1 M sodium phosphate (□) and 0.02 M sodium pyrophosphate/0.2 M sodium phosphate (○) containing: (a) Biliverdin (2.5 μM) and NADPH (100 μM). (b) Biliverdin (2.5 μM) and NADH (700 μM).

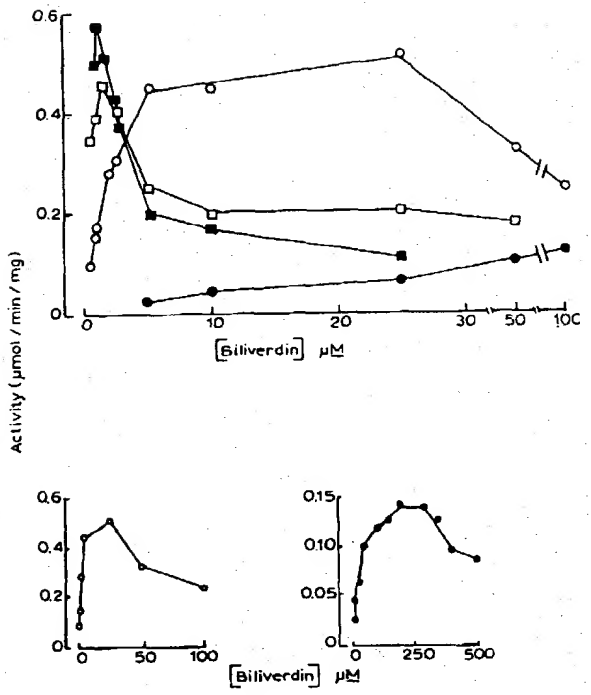

Fig. 2. The effect of pH on biliverdin reductase kinetics. Biliverdin reductase (1 μg) was assayed at 30 °C in a mixture containing NADPH (100 μM) and biliverdin at the concentrations indicated with 0.02 sodium pyrophosphate/0.2 M sodium phosphate: □, pH 5.6, ■, pH 7.5; ○, pH 9; and ●, pH 9.5.

Product inhibition studies

The primary data for all of these experiments are not shown; however, all the inhibition constants can be found in Table II. When inhibition by bilirubin was investigated with biliverdin as the variable substrate at nonsaturating (10 μM) and saturating (100 μM) concentrations of NADPH, mixed inhibition was observed. When the slope and intercept values of the double-reciprocal plots were replotted against the concentration of bilirubin, a linear relationship was obtained and the resultant inhibitor constants K_{ii} (from the intercept replot) and K_{is} (from the slope replot) are shown in Table II. Bilirubin also exhibited mixed inhibition when the variable substrate was NADPH and the bilirubin concentration was held constant at a nonsaturating level (1 μM) or at 10 μM. Unfortunately, it was not possible to saturate the enzyme with biliverdin, as the mechanism becomes complicated by flux through an EBQ complex where B and Q denote biliverdin and NADP, respectively (see below). The 'saturating' concentration of biliverdin (10 μM) was as close to saturation as possible without demonstrating substrate inhibition. When the slope and intercept values were replotted against bilirubin concentra-

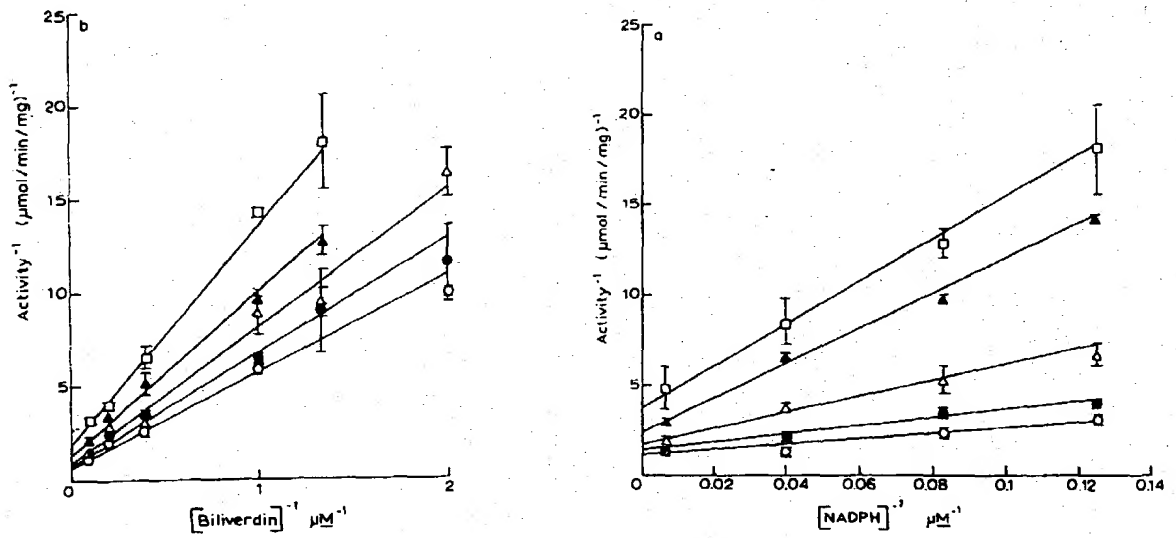

Fig. 3. The initial rate kinetics of biliverdin reductase. (a) The concentrations of biliverdin were 0.75 μM (□), 1 μM (▲), 2.5 μM (△), 5 μM (●) and 10 μM (○). (b) The concentrations of NADPH were 8 μM (□), 12 μM (▲), 25 μM (△), 50 μM (●) and 100 μM (○). All assays were performed in 0.02 M sodium pyrophosphate/0.2 M sodium phosphate (pH 9) with 0.65 μg of biliverdin reductase at 30 °C.

TABLE I

KINETIC CONSTANTS FOR BILIVERDIN REDUCTASE AT pH9

	[NADPH] constant	[Biliverdin] constant
K_a (μM)	12.23 ± 3.93	11.49 ± 5.55
K_b (μM)	6.91 ± 1.53	5.27 ± 1.72
K_{ia} (μM)	9.47 ± 2.12	26.43 ± 12.12
V (μmol/min per mg)	1.31 ± 0.27	1.49 ± 0.47

tion, a linear relationship was obtained and the resultant K_{ii} and K_{is} values are shown in Table II.

NADP showed competitive kinetics with NADPH as the variable substrate at nonsaturating (1 μM) and 'saturating' (10 μM) concentrations of biliverdin (the K_{is} values are shown in Table II). With biliverdin as the variable substrate, NADP showed mixed inhibition at nonsaturating concentrations of NADPH (10 μM), and when the experiment was repeated using saturating concentrations of NADPH (100 μM) the inhibition became uncompetitive. The values for the inhibition constants K_{ii} and K_{is} for both NADP and bilirubin at subsaturating and 'saturating' concentrations of the fixed substrate were calculated from replots of slopes and intercepts against the concentration of the inhibitory product and are listed in Table II.

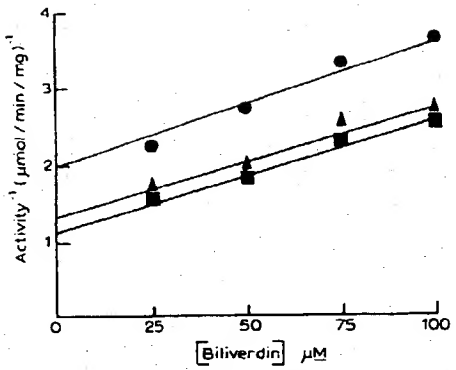

Fig. 4. Substrate inhibition by biliverdin. Dixon plot with NADPH concentrations of 10 μM (●), 25 μM (▲) and 50 μM (■).

Substrate inhibition by biliverdin

The substrate inhibition by biliverdin at pH 9 was further investigated by varying the concentration of NADPH (10–50 μM) and biliverdin (25–75 μM). A Dixon plot of the reciprocal velocity against biliverdin concentration was linear (Fig. 4). The lines at the various concentrations of NADPH were parallel, demonstrating that the substrate inhibition was uncompetitive. The inhibition constant for biliverdin is 48 μM.

Effect of chloride on initial rates

Chloride has a pronounced 'activating' effect on biliverdin reductase activity when NADH is the cofactor; however, no such effect was observed with NADPH at pH 7.2 in the presence of

TABLE II

INHIBITION CONSTANTS FOR BILIVERDIN REDUCTASE

Inhibitor	Variable Substrate	Fixed Substrate Conc. (μM)	K_{is} (μM)	K_{ii} (μM)
Bilirubin	NADPH	1	6.83 ± 2.7	4.93 ± 2.63
		10	5.2 ± 1.35	2.6 ± 0.36
Bilirubin	biliverdin	10	3.91 ± 0.84	6.24 ± 1.69
		100	1.63 ± 0.85	11.17 ± 2.78
$NADP^+$	NADPH	1	84.7 ± 10.74	–
		10	24.3 ± 0.70	–
$NADP^+$	biliverdin	10	690.0 ± 630.0	11.96 ± 5.53
		100	–	90.0 ± 8.8

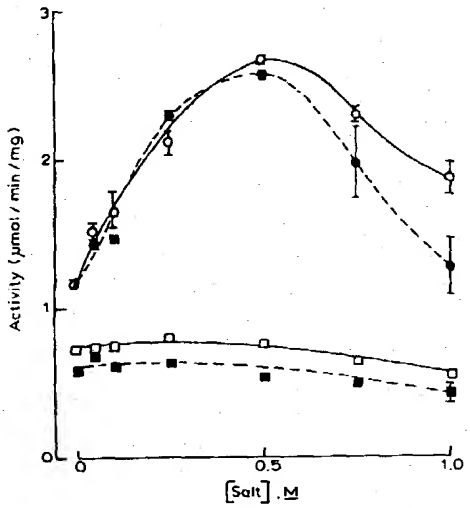

Fig. 5. Effect of chloride on initial rates. Biliverdin reductase (0.35 μg) was assayed in a reaction mixture (2 ml) containing 20 μM biliverdin, 700 μM NADH, 30 μM bovine serum albumin in 0.1 M sodium phosphate (pH 7.2) in the presence of the indicated concentrations of NaCl (●) and KCl (○). The enzyme was also assayed in a mixture containing 2.5 μM biliverdin, 100 μM NADPH in 0.1 M sodium phosphate (pH 8.5) in the presence of the indicated concentrations of NaCl (■) and KCl (□).

bovine serum albumin (Fig. 5). The activation was further investigated by examining the effect of 0.5 M KCl on the initial rate kinetics with biliverdin or NADH as the variable substrate. It can be seen (Fig. 6) that the primary effect of chloride is to abolish the substrate inhibition observed with biliverdin. This effect is mirrored in the NADH kinetics where chloride has little effect on the slope value but increases the apparent turnover number from 1.7 s^{-1} to 5.6 s^{-1}.

Discussion

The initial rate studies indicate that the mechanism obeyed by biliverdin reductase is sequential, as linear intersecting lines were obtained when either substrate was varied at different fixed levels of the other substrate. The mechanism was further investigated by determining the product inhibition patterns [15]. Briefly, since bilirubin is a mixed inhibitor when biliverdin is the variable substrate, Theorell-Chance and rapid-equilibrium random mechanisms are ruled out. A random steady-state mechanism is unlikely as linear product inhibition replots were obtained [15]. These results, taken with our observation that pyridine nucleotides bind to the free enzyme [17], suggest that at pH 9 in the absence of any 'binding protein' the reaction mechanism is ordered. We have been unable to obtain any evidence that biliverdin binds to the free enzyme by analysing difference spectra, by conducting sucrose-density sedimentation experiments in the presence of biliverdin, or by utilising the fact that biliverdin binding to proteins is often accompanied by a distinct spectral change in the presence of a mercaptan [8]. Two minor points concerning the experimental product inhibition patterns and the theoretical patterns for an ordered bi-bi mechanism are worth noting. Firstly, it was not possible to observe the predicted uncompetitive inhibition by bilirubin against NADPH at saturating concentrations of biliverdin as it was not experimentally feasible to saturate with bi-

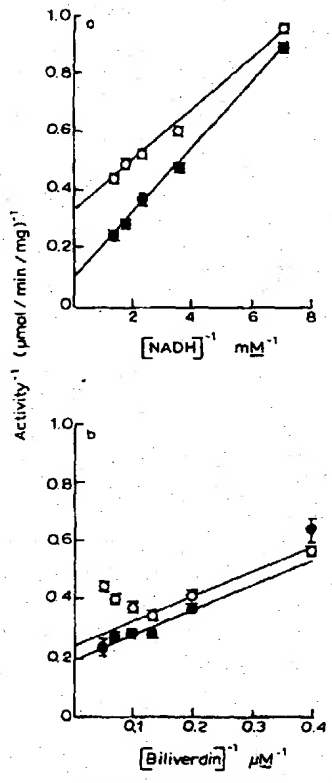

Fig. 6. Effect of chloride on initial rates. Initial rates were measured at pH 7.2 in the absence (○) or presence (●) of 0.5 M KCl. Other conditions are described in the legend to Fig. 5.

liverdin without introducing the complication of substrate inhibition. Secondly, although no inhibition by NADP against biliverdin is predicted at saturating levels of NADPH, significant, albeit reduced, inhibition was observed under these conditions. Cleland [16] has pointed out that in practice one can rarely achieve a high enough concentration of A (NADPH) to negate the effect of Q (NADP) binding. Since NADPH has been shown to bind to biliverdin reductase in the absence of biliverdin [17] it appears that, as with other pyridine-nucleotide linked oxidoreductases, the coenzyme binds before the second substrate [18] in an ordered bi-bi mechanism with bilirubin being the first product to dissociate.

Substrate inhibition studies show that biliverdin inhibited the enzyme uncompetitively at fixed concentrations of NADPH, indicating that biliverdin binds to the enzyme-NADP complex. It should be noted that the substrate inhibition constant increases from approx. 2.5 μM at pH 7.5 to 50 μM at pH 9. It is tempting to ascribe this to deprotonation of a basic residue involved in binding one of the biliverdin propionate side-chains. O'Carra and Colleran [9] have described a reduction in biliverdin reductase activity on esterifying one of the propionate side-chains, although they did not report whether this had an effect on binding. Both results would be compatible with a model where a single salt bridge involving one of the propionate side-chains and a basic residue on the enzyme is less favourable than binding involving two such salt bridges. A similar model will also explain the increase in the apparent K_m for biliverdin as the pH is increased. Dixon plots at high concentrations of biliverdin at pH 9 deviate from linearity to give a limiting reciprocal initial rate (Rigney and Mantle, unpublished data), indicating partial substrate inhibition. It is evident that the enzyme-NADP-biliverdin complex can break down, presumably via an enzyme-biliverdin complex, to regenerate free enzyme. A similar conclusion has been reached at pH 7.2 in the presence of bovine serum albumin [6].

Acknowledgements

This work was supported by the Trinity College Development Fund.

References

1 McDonagh, A.F., Palma, L.A. and Schmid, R. (1981) Biochem. J. 194, 273–282.
2 Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N. and Ames, B.N. (1987) Science 235, 1043–1046.
3 Stocker, R., Glazer, A.N. and Ames, B.N. (1987) Proc. Natl. Acad. Sci. USA 84, 5918–5922.
4 Kutty, R.K. and Maines, M.D. (1981) J. Biol. Chem. 256, 3956–3962.
5 Noguchi, M., Yoshida, T. and Kikuchi, G. (1979) J. Biochem. 86, 833–848.
6 Phillips, O. and Mantle, T.J. (1981) Biochem. Soc. Trans. 9, 275–278.
7 Colleran, E. and O'Carra, P. (1977) in Chemistry and Physiology of Bile Pigments (Berk, P.D. and Berlin, N.I., eds.), pp. 69–80, U.S. Department of Health, Education & Welfare, Publication No. 77–1100, Washington, DC.
8 Rigney, E.M. Phillips, O. and Mantle, T.J. (1988) Biochem. J. 255, 431–435.
9 O'Carra, P. and Colleran, E. (1971) Biochem. J. 125, 110P.
10 Phillips, O., Mantle, T.J., Tuffery, A.R., Heyworth, C.M., Wilson, S.R. and Houslay, M.D. (1984) Biochem. Pharm. 33, 1963–1967.
11 Phillips, O. (1981) Ph. D. Thesis, University of Dublin.
12 McDonagh, A.F. (1979) in The Porphyrins (Dolphin, D., ed.), Vol. 6, pp. 453–455, Academic Press, London.
13 Wilkinson, G.N. (1961) Biochem. J. 80, 324–332.
14 Florini, J.R. and Vestling, C.S. (1957) Biochim. Biophys. Acta 25, 575–578.
15 Cleland, W.W. (1963) Biochim. Biophys. Acta 67, 104–137.
16 Cleland, W.W. (1970) in The Enzymes, Vol. 2, (Boyer, P.D., ed.), pp. 1–65.
17 Rigney, E.M. and Mantle, T.J. (1985) Biochem. Soc. Trans. 13, 502.
18 Dalziel, K. (1975) in The Enzymes, Vol. 11 (Boyer, P.D., ed.), pp. 1–61, Academic Press, New York.